APPENDIX

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1. APPENDIX SUPPLEMENTARY METHODS

Animal care and treatments

Four experimental groups were used: young (6-month-old) control (mef2-73k-$Cre^{-/-}$ $Mfn2^{LoxP/LoxP}$) and Mfn2KO mice (mef2-73k-$Cre^{+/-}$ $Mfn2^{LoxP/LoxP}$) and old control and Mfn2KO mice (22-month-old). For metabolic experiments, 12-month-old mice were also used. Young (4-month-old) MLC1-$Cre^{+/-}$$Mfn2^{LoxP/LoxP}$ mice (SkM-KO mice) were also used. Mice were kept in 12hour dark-light periods in a specific pathogen free (SPF) animal facility, and fed standard chow diet and water *ad libitum*. At appropriate times indicated in figure legends, mice were anesthetized using isofluorane and sacrificed by cervical dislocation. Treatment with antioxidant N-acetylcysteine was performed in 6-month-old mice for three weeks (1% NAC in drinking water). Treatment with chloroquine was performed in 6-month-old mice for five days (intraperitoneal injection twice a day at a dose of 50 mg/kg). Saline was injected to control animals. For Mfn2 overexpression, gastrocnemius muscle was injected with 75 µl of adenovirus encoding for Mfn2 or LacZ as a control at a dose of 2.5×10^8 pfu/muscle. After two weeks, gastrocnemius muscle was extracted. For lifespan analysis, a group of 18 mice of each genotype (control and Mfn2 KO) was used.

Food intake

Mice were placed individually in regular cages and acclimatized for 24h. Food intake was measured for four consecutive days.

In vivo metabolic measurements

Plasma was immediately separated by centrifugation at 4°C and stored at -80°C until assay. A glucose tolerance test (GTT) was performed on mice fasted 16 h overnight. Glucose was measured at time 0, followed by intraperitoneal (i.p.) injection of 2 g/kg glucose. Blood glucose levels were also measured at 5, 15, 30, 60, 90, and 120 min after injection. Plasma insulin levels were measured at 0, 15, 30 and 60 min after injection. An insulin tolerance test (ITT) was performed on mice fasted for 4 h. Glucose was measured at time 0, followed by an i.p. injection of 0.75 U/kg insulin (Humalog, Houten, Netherlands). Blood glucose concentrations were measured at 15, 30, 45, 60 and 90 min after injection. Plasma insulin was determined using the Ultra-Sensitive Mouse Insulin Elisa Kit (Crystal Chem Inc., Downers Grove IL, USA).

Assessment of oxygen consumption in mouse by indirect calorimetry

Mice (n=6 per group) were individually housed in the chamber for 72h with lights on from 6 am to 6 pm and room temperature of 22-24ºC. Mice were acclimated for 24 h and the final 48h period was used for measurements. Animals had free access to food and water during both dark and light cycles. Gas exchange measurements were made every 20 min with room air composed of 20.5% O_2 and 0.05% CO_2.

Measurement of ATP levels in skeletal muscle

ATP levels were determined in gastrocnemius using an ATP determination kit (A22066) from Molecular Probes (Invitrogen) as described previously (Sebastian et al, 2012).

Measurement of ROS, oxidative stress, and antioxidant enzyme activities

H_2O_2 levels were measured in muscle and C2C12 homogenates by using Amplex Red as described previously (Sebastian et al, 2012). Protein oxidation was measured in 5 µg of muscle extracts using the OxyBlot™ Protein Oxidation Detection kit (Chemicon International), according to manufacturer's instructions. Antioxidant capacity was determined in muscle extracts by measuring the activity of superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GP) by commercial enzyme assay kits (Cayman Chemicals), following manufacturer's instructions.

Measurement of glucose oxidation in muscle *ex vivo*

Glucose oxidation was measured in soleus muscle *ex vivo* as described previously (Sebastian et al, 2012).

Evaluation of muscle force and performance

Maximal muscle strength of forelimbs were measured using a grip strength meter (Bioseb). Mice were gently lowered over the top of the grid and pulled back steadily (not jerking) until the grip is released down the complete length of the grid. When the animal releases the grid, the maximal grip strength value of the animal was recorded.

In addition, muscle strength was also measured *ex vivo*. Briefly, mice were sacrificed and soleus muscles were rapidly excised into a dish containing oxygenated Krebs-Ringer solution. Muscle mechanical properties were quantified with the commercially available 1200A isolated muscle test system (Aurora Scientific Inc., ON, Canada). The optimum muscle length (Lo) was determined by administering single electrical pulses while lengthening the muscle until the maximum isometric twitch force on single twitches was attained. Maximum isometric-specific tetanic force (P_o) was determined from the plateau of the curve of the relationship between specific isometric force with a stimulation frequency (Hz) ranging from 1 to 300 Hz for 450 ms, with 2 minutes of rest between stimuli. The twitch force (P_t) and contraction and half-relaxation times ($RT_{1/2}$) were measured during each isometric twitch. The twitch to tetanus ratio (P_t/P_o) was also calculated.

Muscle performance was evaluated by an exhaustion protocol using a treadmill. Prior to exercise performance test, mice were accustomed to the treadmill with a 10-min run at a fixed speed of 14 cm/s once per day for two alternate days. The day of the experiment, each mouse was placed into a lane of the treadmill at an exercise test regimen of 14 cm/s for 8 min and then the speed was increased 2 cm/s every 2 min. Maximum speed was set constant at 46 cm/s. Exhaustion was defined when mice were unable to stand in the treadmill without avoiding repetitive electrical shocks. At that moment, time and distance ran on the treadmill were recorded.

Respiration measurements in permeabilized muscle fibers, isolated mitochondria and muscle cells

Respiration of permeabilized muscle fibers obtained from tibialis muscle and mitochondria obtained from liver (500 µg), WAT (50 µg) and heart (50 µg) was measured at 37ºC by high-resolution respirometry with the Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria) as previously described (Sebastian et al, 2012). Respiration of cultured muscle cells was performed using the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). C2C12 cells were seeded, differentiated, and transduced in XF-24 plates. Respiration was measured in the presence of 5.5 mM glucose and 2 mM glutamine (basal respiration), after addition of 5 µM oligomcyin (leak), 0.5 µM CCCP (maximal respiration) and 1 µM rotenone and 1 µM Antimycin A (non-mitochondrial respiration).

Protein extraction and Western Blotting

Skeletal muscle homogenates for Western Blot analyses were obtained by homogenization of 30 mg of frozen muscle using a polytron in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium ortovanadate, 50 mM NaF, 5 mM sodium pyrophosphate and protease inhibitors cocktail tablet, Roche). Homogenates were incubated for 1h at 4ºC in an orbital shaker and centrifuged at 16,000 g for 15 min and 4ºC to remove cell debris.

Homogenates for Western Blot analyses from cell cultures were obtained by collecting the cells in ice-cold PBS 1X, homogenizing them with a douncer in lysis buffer and centrifuging them at 700 g for 10 min and 4ºC to remove nuclei, cell debris and floating cells.

Mitochondria enriched fractions from muscle or cell culture were obtained by homogenization of 30 mg of muscle or a 10-cm plate of cells using a douncer with a Teflon pestle in homogenization buffer (0.25M sucrose, 50mM KCl, 5mM EDTA, 1 mM sodium pyrophosphate, 5 mM $MgCl_2$, pH 7.4 and protease inhibitors tablet, Roche). Homogenates were centrifuged at 740 g for 5 min at 4ºC. Supernatant was centrifuged at 9,000 g for 15 min at 4ºC. The supernatant was the cytosolic fraction and the pellet (mitochondria enriched fraction) was washed in homogenization buffer and resuspended in lysis buffer.

Nuclear protein extracts were obtained from gastrocnemius using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following manufacturer's instructions.

Proteins from total homogenates, mitochondria, cytosolic or nuclear enriched fractions were solved in 7.5%, 10%, 12.5% or 15% acrylamide gels for SDS-PAGE and transferred to Immobilon membranes (Millipore). The following antibodies were used: Mfn2 (1/1000 Abcam), p62 (1/1000 Progen), NBR1 (1/500 Abcam), LC3 (1/1000 MBL), Porin (1/5000 Abcam), α-Tubulin (1/8000 Sigma), β-Actin (1/5000, Sigma), BNIP3 (1/1000 Abcam), Parkin (1/200 SantaCruz Biothecnologies), HIF1α (1/1000 Cayman), GAPDH (1/1000000 Abcam), TIM44 (1/2000 BD Transduction Laboratories), p-4EPB1 Thr37/46 (1/1000 Cell Signaling), 4EBP1 (1/1000 Cell Signaling), p-S6 Ser235/236 (1/1000 Cell Signaling), S6 (1/1000 Cell Signaling), p-Akt Ser473 (1/1000 Cell Signaling), Akt (1/1000 Cell Signaling), p-AMPK Thr172 (1/1000 Cell Signaling), AMPK (1/1000 Cell Signaling) and K48-linked polyubiquitin (1/1000 Cell Signaling). The antibodies used for the detection of electron transport chain complexes were anti-NADH dehydrogenase (ubiquinone) 1α subcomplex 9 (NDUFA9; complex I), anti-SdhA (complex II), anti-ubiquinol-cytocrome c reductase core protein 2 (Uqcrc2; complex III), anti-COX IV (complex IV), and anti-Atp5a1 (complex V) (1/1000 Life Technologies).

DNA and RNA extraction and real time-PCR

Quantitative real-time PCR was performed using the ABI Prism 7900 HT real-time PCR machine (Applied Biosystems) and the SYBR® Green PCR Master Mix or the Taqman Probes 20X (Applied Biosystems). All measurements were normalized to *β-actin* and *Gapdh*. The following Sybr Green primers were used: *β-actin*, Fwd: GGTCATCACTATTGGCAACGA, Rev: GTCAGCAATGCCTGG; *Gapdh*: Fwd: CATGGCCTTCCGTGTTCCTA, Rev: GCGGCACGTCAGATCCA; *LC3b*, Fwd: AGCTCTTTGTTGGTGTGTAACTGTCT, Rev: TTGTCCTCACAGCTGACATGTATG; *Atrogin1*, Fwd: GCAAACACTGCCACATTCTCTC, Rev: CTTGAGGGGAAAGTGAGACG; *MuRF1*, Fwd: CATTGTGTGACTGGCGATTGT, Rev: TCTCTAGGCCACCGAGTGAGA; *SMART*, Fwd: TCAATAACCTCAAGGCGTTC, Rev: GTTTTGCACACAAGCTCCA; *MUSA1*, Fwd: TCGTGGAATGGTAATCTTGC, Rev: CCTCCCGTTTCTCTATCACG; *FbxO31*, Fwd: GTATGGCGTTTGTGAGAACC, Rev: AGCCCCAAAATGTGTCTGTA; *Bnip3*, Fwd: CACCTTTATCACTCTGCTGAATTCTCT, Rev: GATTTTGTTTTTCATTTCCAGTCTTTTAA; *Bnip3l,* Fwd: AGTTTTGCCATTTTTGAAAGTATTAGTAAA, Rev: GTTTCACCCAACAAGAATCTGTGTA; *p62*, Fwd: CCCAGTGTCTTGGCATTCTT, Rev: AGGGAAAGCAGAGGAAGCTC; *Pdk4,* Fwd: TCAGGTTATGGGACAGACGCT, Rev: CACCAGTCATCAGCTTCGGA; *Mfn2*, For: GTAAGGGCTCGGAGAAGGTATGTG, Rev: TGGCAAGAAGGGAGGCAAGTC.

Transcriptomic analysis

Microarray services were provided by IRB Functional Genomics Core Facility, including quality control tests of total RNA using Agilent Bioanalyzer and Nanodrop spectrophotometry. Briefly, cDNA library preparation and amplification was performed from 25 ng total RNA using WTA2 (Sigma-Aldrich) with 17 cycles of amplification. 8µg cDNA were subsequently fragmented by DNaseI and biotinylated by terminal transferase obtained from GeneChip Mapping 250K Nsp Assay Kit (Affymetrix). Hybridization mixture was prepared according to Affymetrix protocol. Each sample was hybridized to a Mouse Genome 430 PM strip (Affymetrix). Arrays were washed and stained in a Fluidics Station 450 (Fluidics protocol FS450_002) and scanned in a GeneChip Scanner 3000 (both Affymetrix) according to manufacturer's instructions. CEL files were generated from DAT files using GCOS software (Affymetrix).

Annotation of Affymetrix microarray was performed using Bioconductor R package *mouse4302.db* (Affymetrix Mouse Genome 430 2.0 Array annotation data (chip mouse4302)). Differential expression analysis was carried out using GaGa (GaGa hierarchical model for high-throughput data analysis. R package version 2.10.0. David Rossell, 2013). Data was previously corrected by technical factors (scanning date) and familiar relationship between individuals using a linear model. Genes were classified according to their expression profile in the different conditions (old-young, Mfn2 KO - control) using a threshold of 5% for FDR and 1.20 fold-change in absolute value. Relevant results for differential expression analysis were represented in a heatmap showing expression values centered and scaled across samples. In the heatmap, samples and probesets were represented under a hierarchical clustering in which correlation distance and average linkage method was used. A gene was considered as differentially expressed when at least one of its probeset was called as differentially expressed. Genes showing probesets differentially expressed in the opposite direction were excluded from the results reported at the gene level. Pathway enrichment was assessed through Geneset Enrichment Analysis (GSEA). GSEA was applied to the differential expression analysis between young and old control mice and between Mfn2KO and control young mice. In these analyses, we used the curated list of genesets included in the Broad Institute Molecular Signature Database (MsigDB-C2). Genes in MsigDB-C2 were translated to *Mus musculus* homolog genes using annotation from the Mouse Genome Informatics (MGI) database. The complete dataset was deposited to the National Center for Biotechnology Information's Gene Expression Omnibus Database, and is accessible through GEO Series accession number GSE71501.

Proteasome activity

Briefly, 100 µl of ice-cold PBS with 5mM EDTA were added to 1mg of muscle and the sample was then sonicated. Tissue lysates were centrifuged at 13.000g for 5 min at 4ºC and supernatants were subjected to protein quantification and diluted until 0.2mg/ml. 50 µl corresponding to 10 µg of total protein were added to 50 µl of the luminescent reagent containing the Ultra-Glo Luciferase and the signal peptides for chymotrypsin-like, trypsin-like and caspase-like activities (Promega). To calculate proteasomal activity, dual measurements with or without specific proteasomal inhibitor adamantane-acetyl-(6-aminohexanoyl)$_3$-(leucinyl)$_3$-vinyl-(methyl)-sulfone (AdaAhx$_3$L$_3$VS) (Calbiochem) were carried out. After mixing all the components and upon completion of 1h preincubation, the resulting luminescence was measured.

Mitochondrial DNA copy number

Total DNA was amplified with specific oligodeoxynucleotides for mitochondrial DNA or SdhA (nuclear gene). The primers to amplify cytochrome c oxidase subunit II in mitochondrial DNA were as follows: Fwd CTACAAGACGCCACAT, and Rev GAGAGGGGAGAGCAAT. Other primers used to amplify mouse mitochondrial DNA in positions 1,212 and 1,352 were: Fwd ACCGCAAGGGAAAGATGAAAG, and Rev AGGTAGCTCGTTTGGTTTCGG. The primers used to amplify nuclear DNA (mSDHA) were: Fwd TACTACAGCCCCAAGTCT, and Rev TGGACCCATCTTCTATGC. We calculated the mitochondrial DNA copy number per cell using Sdha amplification as a reference for nuclear DNA content.

Electron microscopy

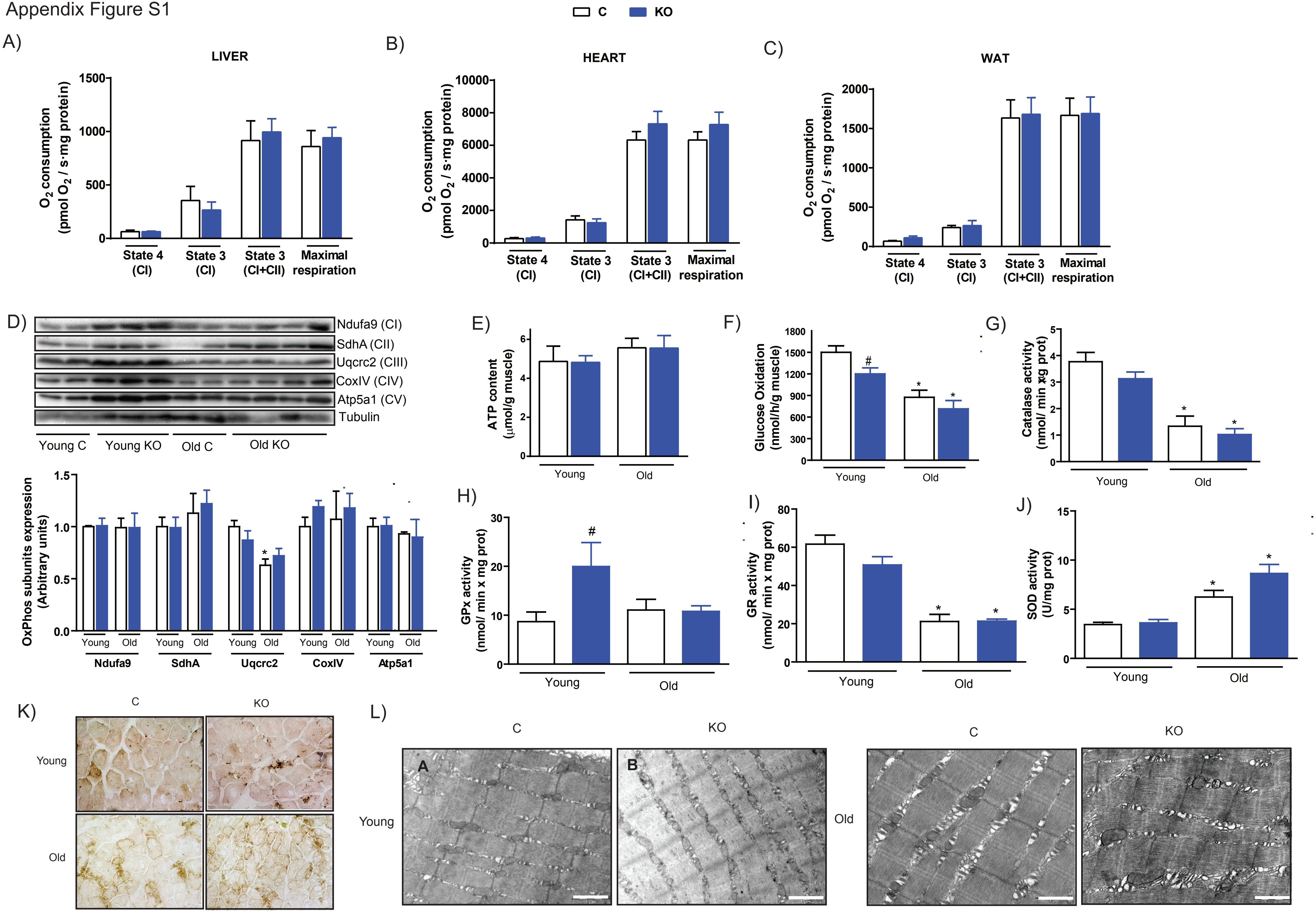
C2C12 myotubes were washed twice with 0.1 M phosphate buffer at room temperature. For fixation, cells were incubated in 2.5% glutaraldehyde solution at room temperature for 70 min. Following post-fixation in 1% osmium tetroxide in 0.1 M phosphate buffer at 4ºC for 2 h, cells were washed with highly pure water and kept overnight in 0.1 M phosphate buffer. Quadriceps muscles were cut into pieces of about 1mm^3 and transferred to glass vials filled with 2% parafomaldehyde and 2.5% glutaraldehyde in phosphate buffer. They were kept in the fixative solution for 24 h at 4ºC. Then, they were washed with the same buffer and post-fixed with 1% osmium tetroxide in the same buffer containing 0.8% potassium ferricyanide at 4ºC. Next, the samples were dehydrated in acetone, infiltrated with Epon resin for 2 days, embedded in the same resin orientated for longitudinal sectioning and polymerized at 60ºC during 48 hour. Semithin sections were made in order to corroborate that the orientation was good under the light microscope. When found, ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems, Vienna, Austria) and mounted on Formvar-coated copper grids. They were stained with 2% uranyl acetate in water and lead citrate. Then, sections were observed under a JEM-1010 electron microscope (Jeol, Japan) equipped with a CCD camera SIS Megaview III and the AnalySIS software.

2. APPENDIX SUPPLEMENTARY REFERENCE

Sebastian D, Hernandez-Alvarez MI, Segales J, Sorianello E, Munoz JP, Sala D, Waget A, Liesa M, Paz JC, Gopalacharyulu P, Oresic M, Pich S, Burcelin R, Palacin M, Zorzano A (2012) Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 5523-5528

3. APPENDIX FIGURES

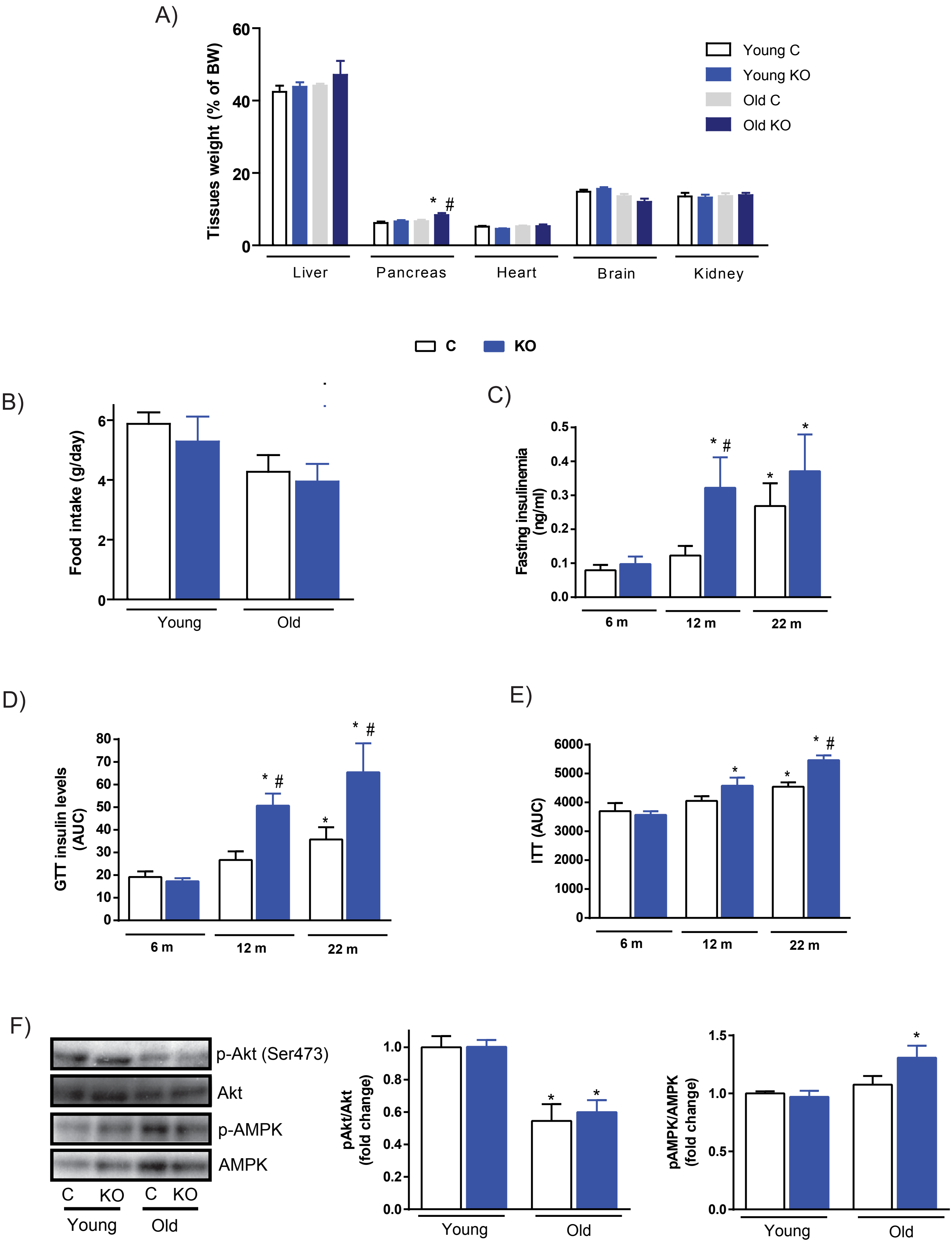
Appendix Figure S1



APPENDIX FIGURE S1. OXPHOS protein expression, ATP levels, glucose oxidation, and antioxidant enzyme activity in muscle from young and old control and Mfn2KO mice

A-C) Oxygen consumption was assessed in mitochondrial fractions obtained from liver (A), heart (B) and white adipose tissue (C) in old control and Mfn2KO mice (n=6 mice per group). D) Protein expression of different OXPHOS subunits was measured in gastrocnemius muscle from young and old control and Mfn2KO mice (n=6 mice per group). Data were normalized by tubulin as a loading control and expressed as a fold change compared to young control mice. E) ATP content was measured in gastrocnemius muscle from young and old control and Mfn2KO mice (n=6 mice per group). F) Glucose oxidation was measured *ex vivo* in soleus muscle from young and old control and Mfn2KO mice (n=6 mice per group). G-J) Antioxidant enzyme activities (catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase) were measured in gastrocnemius muscle from young and old control and Mfn2KO mice (n=5 mice per group). K) Representative image of sequential COX/SDH staining performed in gastrocnemius muscle from young and old control and Mfn2KO mice (n=4 mice per group). L) Representative TEM images of longitudinal sections of quadriceps muscle from young and old control and Mfn2KO mice (scale bar: 1 μm). Data represent mean ± SEM. #$p<0.05$ Mfn2KO vs. control mice, *$p<0.05$ old vs. young mice.

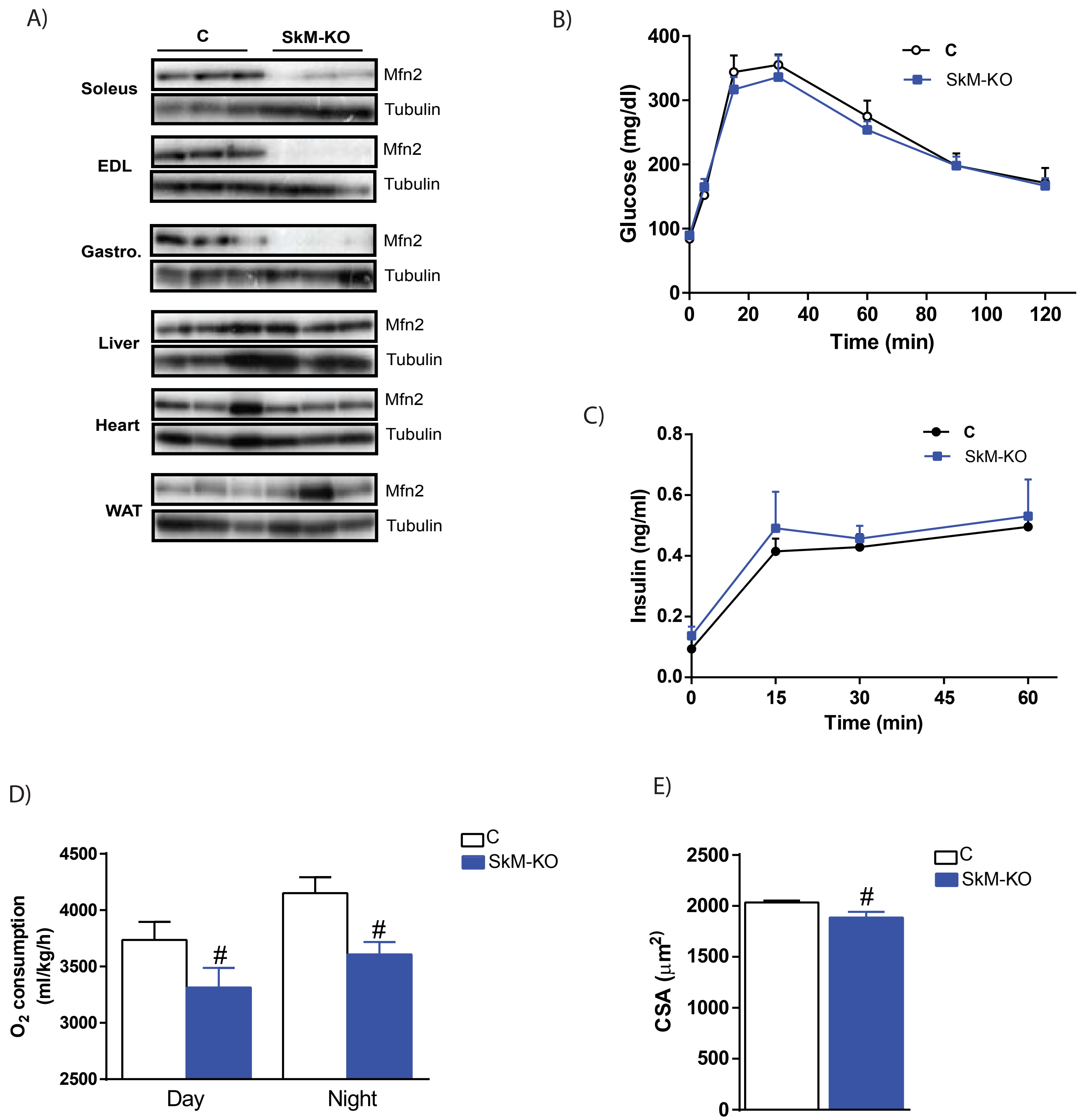
Appendix Figure S2



APPENDIX FIGURE S2. Mfn2KO mice exhibit an accelerated age-related metabolic disease without changes in food intake

A) Weight of different tissues wasS measured in young and old control and Mfn2KO mice (n=6 mice per group). B) Food intake was determined in young and old control and Mfn2KO mice (n=6 mice per group). C-E) Different metabolic parameters (fasting insulinemia, insulin levels during a GTT and ITT) were determined in young, middle aged and old control and Mfn2KO mice. (n=6 mice per group). F) Phosphorylation of Akt and AMPK was evaluated in gastrocnemius muscle from young and old control and Mfn2KO mice (n=6 mice per group). Data were expressed as a fold change compared to young control mice. Data represent mean ± SEM. #$p<0.05$ Mfn2KO vs. control mice, *$p<0.05$ old vs. young mice.

Appendix Figure S3



APPENDIX FIGURE S3. Muscle-specific Mfn2KO (SkM-KO) recapitulate the main phenotypic features of young Mfn2KO mice.

A) Mfn2 protein levels were measured by western blot in different tissues from 4 month-old SkM-KO mice (MLC1-Mfn2KO mice, n=3 mice per group). B) Glucose tolerance test (GTT) was performed in control and SkM-KO mice (n=6 mice per group). C) Insulin levels were measured in plasma from control and SkM-KO mice subjected to GTT (n=6 mice per group). D) Oxygen consumption was assessed by indirect calorimetry in control and SkM-KO mice (n=4 mice per group). E) Cross sectional area (CSA) was quantified from transversal sections of gastrocnemius muscle from control and SkM-KO mice (n=3 mice per group, 200 fibers per mouse). Data represent mean ± SEM. #p<0.05 SkM-KO vs. control mice.